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CYTOKINE COMPOSITION AND METHOD OF ITS PRODUCTION

This application claims priority to U.S. patent application Serial No. 09/595,338, filed June 14, 2000, which is a conversion application of U.S. provisional application Serial No. 60/139,313, filed June 15, 1999. Both applications are incorporated by reference herein.

Field Of the Invention

The present invention relates to a complex cytokine composition for use in treating viral infections, cancers and other conditions responsive to cytokines.

References

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Sambrook J *et al.*, in MOLECULAR CLONING: A LABORATORY MANUAL, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, Vol. 2, 1989.

Background Of the Invention

Cytokines are useful in treating a number of human pathologies, including cancers viral infections, and inflammation. Typically, cytokine treatment involves administering a single, isolated cytokine, e.g., interferon-alpha (IFN-alpha), interferon-beta (IFN-beta) tissue necrosis factor (TNF), etc. Although some treatment efficacy is achieved, the extent of improvement may be suboptimal, particularly in view of severe side effects which the patient may experience

Since cytokine-producing cells in the body, such as monocytes, macrophages, B cells, dendritic cells, T_H1 and T_H2, mast cells, NK cells and bone-marrow stromal cells, produce complex mixtures of cytokines in their disease-fighting role, it is not surprising that a single cytokine, when administered alone, and at high concentrations, are not optimally effective.

It would therefore be desirable to prepare, for clinical use, a composition that contains a complex mixture of cytokines, preferably in a ratio that the body recognizes as a natural mixture of cytokines. It would be particularly desirable to produce such a cytokine mixture in a cell culture system that produces and secretes high levels of cytokines.

Parent application SN 09/595,338 and its provisional predecessor application disclose a cell culture method of making high levels of cytokines, by growing cytokine-producing human cells under conditions of PKR-overproduction (PKR here refers to dsRNA-dependent protein kinase), inducing cytokines, e.g., by addition of polyIC or viral dsRNA, and harvesting cytokines produced and secreted by the cells in the cell medium. The present application is concerned with cytokine compositions produced by this method, to methods of obtaining improved cytokine compositions, and to methods of use of the compositions.

Summary of the Invention

The invention includes, in one aspect, a composition containing a mixture of human cytokines produced by culturing a human cell line (i) capable of producing cytokines, and (ii) transformed with a PKR gene, in a culture medium effective to cause overproduction of PKR in the cell line. The PKR-overproducing cell line is treated to induce cytokine production, e.g., by the introduction of polyIC or viral dsRNA, and cytokines produced by the cells and secreted into the

medium are isolated, e.g., removing unwanted components from the cell culture medium.

The composition is preferably produced by fractionating the collected cytokines to isolate cytokines having selected properties, such as binding affinity to binding agents such, as antibodies or receptors, or a selected molecular weight range, or range of isoelectric points.

For use in tumor treatment, the composition preferably include two or more cytokines selected from among IL-1-alpha, IL-1-beta, IL-2, IL-4, IL-6, IL-12, IFN-alpha, IFN-beta, IFN-gamma, oncostatin, TNF-alpha, TNF-beta, GM-CSF, G-CSF, and M-CSF, more preferably selected from among IL-2, IL-12, IFN-alpha, IFN-beta, TNF-alpha, and GM-CSF. The isolating step may include removing from the composition, cytokine(s) selected from among IL-3, IL-5, IL-7, IL-8, IL-9, IL-10, IL-11, IL-1 and TGF-beta.

For use in treating viral infection, the composition preferably includes two or more cytokines selected from among IFN-alpha, IFN-beta, IFN-gamma, IL-3, IL-7, IL-8, IL-12, and GM-CSF, more preferably selected from among IFN-alpha, IFN-beta, IL-8, IL-12, and GM-CSF. The isolating step may include removing from the composition, cytokine(s) selected from among IL-1, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-11, IL-13, TNF-alpha, TNF-beta, TGF-beta, and oncostatin.

For use in treating an inflammatory condition, the composition preferably includes two or more cytokines selected from among IL-4, IL-5, IL-6, IL-10, IL-11, IL-13, and IFN-gamma, and preferably selected from among IL-4, IL-5, IL-6, and IL-10. The isolating step may be include removing from the composition, cytokine(s) selected from among IL-1, IL-2, IL-3, IL-7, IL-8, IL-9, IL-12, TNF-alpha, TNF-beta, TGF-beta, and oncostatin.

In another aspect, the invention includes producing a mixture of cytokines, for use as a therapeutic composition. The method includes the steps (a) culturing a human cell line (i) capable of producing cytokines, and (ii) transformed with a PKR gene, in a culture medium effective to cause overproduction of PKR in said human cell line; (b) treating the PKR-overproducing cell line to induce cytokine production; and (c) isolating a mixture of at least two cytokines produced by the cell line and secreted into culture medium.

The cell line used in the method may be additionally transfected with a

gene that expresses a protein effective to inhibit apoptosis in the cell line, to enhance the viability of the cells during cytokine production. The cultured cells may also be primed, prior to cytokine induction, using a priming agent such as PMA, calcium ionophores, sodium butyrate, endotoxin, and cytokines.

To simplify the cytokine isolation step(s), the cell culturing may be carried out in serum-containing medium, but with the inducing and isolating steps being carried out in medium that is substantially serum free. The isolating step may include contacting culture medium containing secreted cytokines with a solid support having surface-attached antibodies specific against the cytokine(s) to be isolated, washing the solid support to remove non-bound material, and eluting the cytokines specifically bound to the support.

For producing a cytokine composition useful in the treatment of cancer, the cultured cell line used in the method is preferably derived from a parental B-cell or monocyte cell line; for a cytokine composition useful in treating viral infection, from a parental B-cell or fibroblast cell line; and for an anti-inflammatory composition, from a parental T-cells.

Also disclosed are methods of treating cancer, viral infection and inflammatory conditions with the respective cytokine compositions above.

These and other objects and features of the invention will become more fully apparent from the following detailed description of the invention.

Detailed Description of the Invention

I. Definitions

The term "vector" refers to a nucleotide sequence that can assimilate new nucleic acids, and propagate those new sequences in an appropriate host. Vectors include, but are not limited to recombinant plasmids and viruses. The vectors (*for example*, plasmid or recombinant virus) employed in the method of the invention include, in general, a DNA encoding a desired protein, a promoter which controls the expression of the protein-encoding DNA, and other appropriate control sequences, e.g., a terminator sequence, as well as elements needed for transfection, replication, and selection, e.g., selectable marker genes.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism.

The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

"Promoter sequences" encode either constitutive or inducible promoters. The promoters may be either naturally occurring promoters or hybrid promoters. Hybrid promoters combine elements of more than one promoter, are generally known in the art, and are useful in practicing the present invention.

"Cells transfected with a vector" refers to cells which have been exposed to a vector, and have taken up the vector, either as a self-replicating genetic element or by integration into the cell genome, in a manner that allows expression of the protein(s) encoded by the transfecting vector. The expression may be under the control of a constitutive promoter in the vector, in which case protein expression occurs in the absence of an inducing agent in cells in culture, or under the control of an inducible promoter, requiring the presence of an inducer in the culture medium in order to achieve expression or high levels of expression of the vector gene.

"PKR" refers to dsRNA-activated protein kinase (PKR), also referred to as P1/e1F2 kinase, DAI for dsRNA-activated inhibitor, and p68 (human) kinase, is a serine/threonine kinase whose enzymatic activation requires binding to dsRNA or to single-stranded RNA presenting internal dsRNA structures and consequent autophosphorylation (Galabru and Hovanessian, 1987; Meurs, *et al.*, 1990). PKR play a key role in the expression of a number of useful cytokines including interferons, as described in WO 97/08324, expressly incorporated by reference herein.

As used herein, the term "PKR expression" refers to transcription and translation of a PKR gene, the products of which include precursor RNAs, mRNAs, polypeptides, post-translation processed polypeptides, and derivatives thereof, including PKRs from other non-human species such as murine or simian enzymes.

By "over-expression of a cytokine-regulating factor", e.g., PKR, is meant higher than normal levels of cytokine-regulating factor activity. "Normal" cytokine-regulating factor activity or expression is reported as a range of cytokine-

regulating factor activity or expression, which is generally observed for a given type of cells which have not been transfected with a vector encoding the cytokine-regulating factor, are unstimulated (not induced or primed) and uninfected. It will be understood that the range of normal cytokine-regulating factor activity will vary dependent upon the particular factor, cell type and for a given cell type may vary somewhat dependent upon culture conditions.

Higher than normal level preferably means at least 150%, more preferably at least 200 or 300%, most preferably at least 500%, of the normal level for a given cytokine-regulating factor under the particular culture conditions employed. The cytokine-regulating factor-overexpressing cell culture may be constitutive for over-expression of the cytokine-regulating factor or inducible for over-expression of the cytokine-regulating factor.

"Proteins that inhibit apoptosis" refers to proteins that, when expressed at elevated levels in a cell, inhibit apoptosis, and in particular, apoptosis associated with PKR overexpression and/or cytokine induction. Proteins effective to inhibit apoptosis may be, for example, Bcl-2a, Bcl-X_L, a modified from of eukaryotic translation initiation factor 2 alpha (eIF-2 alpha) and eukaryotic translation initiation factor (eIF-3), a modified form of Fas-associated death domain (FADD), a modified form of Bcl-X_S, a modified form of Bcl-2-homologous antagonist/killer (BAK) and a modified from of BAX, preferably Bcl-2a or Bcl-X_L.

Similar definitions as above apply to cells that overexpress a protein capable of inhibiting apoptosis, such as Bcl-2, Bcl-X_L and related homologues. Thus, "overexpression" of Bcl-2 or Bcl-X_L, respectively means a range of Bcl-2 or Bcl-X_L activity or expression which is greater than that generally observed for a given type of cells which are not transfected with a vector encoding Bcl-2 or Bcl-X_L, and have not been stimulated to undergo apoptosis.

"Cytokines" refers to a group of low-molecular-weight regulatory proteins that regulate the intensity and duration of the immune response by exerting a variety of effects on lymphocytes and other immune cells. A list of cytokines that have been characterized to date is given in Table 1 below.

"Cytokine-producing cells" refers to cells, typically blood cells, that secrete cytokines in vivo, and also in cell culture. Such cells include monocytes, macrophages. B cells, endothelial cells T_H1 and T_H2 (T-helper cells) NK (natural

killer) cells, mast cells, bone-marrow stromal cells, T_C cells, eosinophils, leukocytes, fibroblasts, thymic epithelial cells, and platelet cells.

II. Method of the Invention

The method of the invention is intended for producing a composition containing a mixture of cytokines for therapeutic uses, and in particular, a cytokine compositions for use in treating cancer, viral infection, and inflammation, as detailed in Section III below.

Selection of cells. The method uses human-cytokine producing cells which are selected for their ability to produce a desired mixture of cytokines, e.g., a mixture suitable for cancer treatment, treating viral infection, or treating inflammation. Table 1 below gives known cellular sources for most cytokines, and this table can be used in selecting appropriate cells for producing desired mixtures of cytokines. Cell lines derived from these cells are suitable candidates for use in producing desired cytokine mixtures.

Table 1. Human cytokines and their cellular sources

Cytokine	Miol Wt	Major cellular source
INTERFERONS		
IFN-α- (>12 subtypes)	16-20	macrophage, fibroblast, lymphoblastoid cell
IFN-β	20	fibroblast, macrophage, epithelial cell
IFN-γ	20-25	T-cell, NK cell
TUMOR NECROSIS FACTORS		
TNF-α	17	monocyte/macrophage, fibroblast, T-cell
TNF-β (lymphotoxin)	25	T-cell, B-cell
INTERLEUKINS		
IL-1α & IL-1β	17.5	monocyte/macrophage, endothelial cell fibroblast, keratinocyte
IL-2	15-17	T-cell
IL-4	15-19	T-cell, mast cell
IL-5	50-60	T-cell, mast cell
IL-6	26	monocyte/macrophage, fibroblast, T-cell
IL-7	25	bone marrow cells
IL-8	6-8	monocyte, fibroblast, chondrocyte endothelial cell, keratinocyte
IL-9	32-39	T-cell
IL-10	19	T-cell
IL-11	23	bone marrow cells
IL-12 p35 & p40	35,40	B-cell, lymphoblastoid cell
COLONY STIMULATING FACTORS		
IL-3	20-26	T-cell, mast cell
G-CSF	20	monocyte/macrophage, fibroblast,endothelial cell
GM-CSF	22	T-cell, fibroblast,endothelial cell
M-CSF	70-90	monocyte, fibroblast,endothelial cell

GROWTH FACTORS		
Epidermal growth factor	6	macrophage
Fibroblast growth factors (acidic & basic)	14-18	platelet, macrophage, endothelial cell
Platelet-derived growth factors 1 & 2	14-18	platelet, monocyte/macrophage endothelial cell
Transforming growth factor-α	5-8	macrophage
TGF-β	25	platelet, monocyte/macrophage, T-cell, fibroblast,endothelial cell
INTERCRINES		
Macrophage inflammatory proteins 1α , 1β	8	monocyte, fibroblast, T cell
RANTES	9.5	T-cell

Human cytokine-producing cells suitable for producing a mixture of cytokines suitable for use in treating cancer (including solid tumors, melanomas, leukemias, and other types of cancers or neoplasms) include B-lymphocytes (B-cells), monocytic cells, and T helper cells. Examples of isolated B-cell parent cell lines that are suitable for in vitro culturing are Namalwa cells. Suitable monocytic parent cell lines are U937 and THP-1 cells. T cells, including T-helper cells 1 and 2 (T_H1, T_H2) that are available for in vitro culturing include Jurkat, CEM cells.

Human cytokine-producing cells suitable for producing a mixture of cytokines suitable for use in treating viral infection (including infection by HIV, hepatitis viruses, such as HBV, and HCV virus, and other human-pathogenic viruses) include, generally B-lymphocytes (B-cells) and fibroblast cell lines. Examples of isolated B-cell parent cell lines that are suitable for in vitro culturing are given above. Suitable fibroblast parent cell lines are MRC5, HFF, and WI-38 cells.

Human cytokine-producing cells suitable for producing a mixture of cytokines suitable for use in treating inflammation (including asthma, allergies, and rheumatoid arthritis) generally T-cells, including T-helper cells 1 and 2 as exemplified above.

Examples of other appropriate clonal cell lines for expression of a cytokine-regulating factor include, but are not limited to Vero, Flow 1000 cells, Flow 4000 cells, FS-4 and FS-7 cells, MG-63 cells, and CCRF-SB cells. More generally, appropriate primary cell types for expression of a cytokine-regulating factor include, but are not limited to cells of the monocyte/macrophage lineage, T-cells, mast cells, fibroblasts, bone marrow cells, keratinocytes, endothelial and epithelial cells, platelets and various other immune system cells.

In general, U937 cells are preferred for production of FGF and sTNF-R; Jurkat cells are preferred for production of IL-3 and TNF-beta; fibroblasts are preferred for production of FGF and angiostatin; U937 cells are preferred for

production of TNF-alpha, IFN-alpha, IL-6 and homologs thereof; CD-4 expressing cells including Jurkat and HUT are preferred for production of TNF-beta; and T and B-cells including Jurkat and Namalwa are preferred for production of IL-8 and homologs thereof.

Transfection of cells for PKR overexpression. The parent cells are prepared for use in the method of the invention by transfection with a PKR vector containing a PKR gene under the control of a constitutive or inducible promoter, such that culturing the cells in a suitable growth medium leads to overexpression of the PKR gene. The cells may also be transfected with a second vector that expresses a protein that inhibits apoptosis in cells under conditions of PKR-overexpression and/or cytokine production.

Examples 1 and 2 below describe exemplary vectors and transfection methods for obtaining human cytokine-producing cells suitable for use in the invention. Typically, the cells are first transfected with the vector containing the anti-apoptotic gene, then successful transformants are further transfected with the vector containing the PKR gene. This allows for the second transfection and selection to be carried out with cells that have already been "stabilized" with an anti-apoptopic function. The vector construction and transfection conditions are conventional, and known to those skilled in the art. In particular, it is well known, in such vector constructions, to obtain suitable plasmids or other vectors, e.g., from commercial sources, capable of being introduced into and replicating within selected human cells, where the plasmids may also be equipped with selectable markers, insertion sites, and suitable control elements, such as termination sequences. Typical coding sequences for a PKR gene, and for a Bcl-X_L gene are referenced in Example 1, and can be obtained from the GenBank as cited.

Appropriate cloning and expression vectors for use in human cells are also described in Sambrook *et al.* (1989) Molecular Cloning: A Laboratory Manual (Second Edition), Cold Spring Harbor Press, Plainview, N.Y. and Ausubel FM *et al.* (1989) Current Protocols in Molecular Biology, John Wiley & Sons, New York, N.Y., expressly incorporated by reference herein. Exemplary promoters include both constitutive promoters and inducible promoters, examples of which include a CMV promoter, an SV40 early promoter, an RSV promoter, an EF-1alpha

promoter, a promoter containing the responsive element (TRE) and the metallothienein promoter.

Although the invention preferably employs cytokine-producing cells that overproduce PKR by virtue of PKR-gene transfection, the invention also contemplates culturing non-transformed cells, or cells transformed with an apoptosis-inhibiting gene only, under conditions which produce above-normal levels of endogenous PKR, e.g., by addition to the medium or priming or other activating agents. Additional approaches include inactivation or decreasing the levels of the PKR-inhibiting factor, p58 which normally inhibits PKR activity. Mutation, modification or gene-targeting ablation of p58 will result in enhanced PKR activity (Barber, G.N. *et al.*, 1994).

Another example includes natural, synthetic or recombinant activators of PKR that can enhance the expression of PKR, *for example* the PKR activator protein, PACT (Patel, R.C. and Sen, G.C., 1998).

Culture conditions. The transfected human cells are cultured under conditions of PKR overexpression. The culture medium itself is a standard medium containing physiological salts, nutrients, such as standard RPMI, MEM, I<EM. DMEM media supplemented with 5-10% serum, such as fetal bovine serum. Culture conditions are also standard, typically carried out at 37°C, and in stationary or roller cultures for typically 24-48 hours, or until desired levels of PKR are achieved. If the PKR gene is under the control of an inducible promoter, the inducing agent, e.g., a metal salt, is added to the medium at a concentration effective to induce high-level PKR expression. Culture conditions suitable for a number of cytokine-producing cells, such as fibroblasts, B-cells, T-cells, endothelial cells, dendritic cells, and monocytes are available from the published literature. Preferred media for various types of cytokine-producing cells are given below. An exemplary medium and culture conditions are give in Example 3.

The activity of PKR expression can be determined by methods known in the art. Assays for PKR expression include autophosphorylation assays, assay for eIF2 alpha phosphorylation, Western and Northern blot analysis and reverse transcriptase polymerase chain reaction (RT-PCR) for PKR mRNA.

During this cell-culturing step, the cells may further be exposed to a priming agent to further enhance the inducible levels of cytokines produced by the

cells. Priming is carried out by treating the cells with a priming agent, *for example*, phorbol myristate acetate (PMA) and other phorbol esters, calcium ionophores, interferon-α, interferon-γ, interferon-beta, G-CSF, GM-CSF, PDGF, TGF, EGF or chemokines (IL-8, MCP or MIP), sodium butyrate, a kinase activator or a transcription activator. Suitable priming agent concentration may be found from literature references. For example, a concentration of PMA in the range 5-50 nM, preferably about 10 nM, is suitable.

After culturing the cells under conditions of PKR overproduction (with or without priming), and with above-normal levels of PKR in the cells, the cells are then treated to induce cytokines. Preferred inducing agents are dsRNA, preferably polyIC (poly r(I):poly r(C)), or viral dsRNA such as Sendai virus RNA, administered for example by viral infection or exposure to killed virus or isolated viral dsDNA. In addition, cytokine induction may be produced or enhanced by adding particular cytokines known to stimulate cytokine production in certain cells, as set out in Table 1. . The inducing agent is added in an amount effective to induce cytokine production, e.g., effective to obtain stimulated levels of cytokines produced and secreted into the culture medium, e.g., in the concentration range between 5-50 ug/ml.

After addition of the inducing agent, the cells are further incubated until desired levels of induced and secreted cytokines are obtained. Incubation at 37°C for at least 12-48 hours, and up to 72-96 hours are suitable.

At various time points following induction of cytokines, the cell medium may be tested for the presence or one or more selected cytokines. The presence of selected cytokines may be assayed by direct detection, e.g., with an antibody binding assay (see below) or indirectly by the effect of the culture medium on the activity of various cytokine-responsive cells (Table 1), according to well known biological assays.

In one preferred method, the culturing step is carried out in culture medium containing serum, and the induction step is carried out in medium which is substantially serum free. This approach is advantageous in that the final cell culture medium from which the cytokine mixture is obtained will have a minimum contamination by added serum proteins, and thus will lend itself to simpler

purification methods for obtaining a cytokine composition suitable for administration to humans.

Isolation of Cytokines. After cytokine induction for an appropriate culture period, the cell medium containing the cell-produced and secreted cytokines is harvested, and the further treated to isolate desired cytokines for a final cytokine composition. To the extent that the harvested cell medium contains suspended cells, the medium may be centrifuged at low speed, filtered, or otherwise treated to remove cells and cellular debris. The medium may be further treated, e.g., by diafiltration or molecular sieve chromatography, to remove low molecular weight components, such as pyrogens, and higher molecular weight components that are outside the molecular weight range of cytokines, which is about 10-40 kdaltons.

To obtain a desired cytokine mixture, such as set out below, the culture medium from one or more cytokine-producing cells is subjected to various protein isolation procedures, such as antibody-affinity column chromatography, ion exchange chromatography; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; and gel filtration using, for example, Sephadex G-75. Various methods of protein purification may be employed and such methods are known in the art and described for example in Deutscher, Methods in Enzymology, 182, 1990; Scopes, Protein Purification: Principles and Practice, Springer-Verlag, New York, 1982. The purification step(s) selected will depend, for example, on the nature of the production process used and the particular cytokine produced.

In a preferred isolation method, a composition containing a desired cytokine mixture is prepared by isolating the selected cytokines by affinity chromatography. The method employs, as the affinity medium, purified anti-cytokine antibodies or cytokine-specific receptors that bind to desired cytokines. Antibodies specific against a number of cytokines are available commercially from Sigma Chemical Co., Sigma Catalog 2000-2001, p.1185. (St. Louis, MO) and other vendors of biological materials.

An affinity column that is suitable for cytokine-antibody interaction can be obtained from commercial vendors, e.g. Pharmacia. The column is equilibrated

with solutions such as Tris-buffered saline (TBS). Selected antibodies to the desired cytokines in the composition are loaded on a pre-equilibrated affinity column to allow the binding of the antibodies. The cell medium, either as obtained or after certain partial purification procedures, is chilled to low temperature, e.g., 0 C, and loaded onto the affinity column. The column containing the mixture of the cytokines and their antibodies are then incubated on a tumbling device at room temperature for 12- 18 hours to allow binding between the cytokines and their antibodies.

After incubation, the column is washed with one or more washing solutions, such as TBS, to wash away unbound cytokines and other unwanted material in the cell medium. The bound cytokines are then eluted with an appropriate eluting buffer (Sambrook J, *et al.*, in MOLECULAR CLONING: A LABORATORY MANUAL, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, (1989)). U.S. Patent Nos. 4,385,991, 3,983,001, 4,937,200, and 5,972,599 are all provided details on the use of affinity chromatography for protein purification.

The chromatographic separation may be carried out by successively removing a single selected cytokine from which of a plurality of different affinity columns, and combining the resultant individually purified cytokines, or more preferably, by mixing the cell medium with an affinity column material that contains a column plurality of attached antibodies against the several cytokines to be included in the composition.

The cytokine composition of the invention includes at least two, and preferably three of more cytokines produced by one or more cytokine-producing cells. The cytokines in the mixture are selected from their particular treatment activity, and in particular, for their roles or potential benefit in treating cancer, viral infection, and inflammation. According to another important aspect of the composition, the cytokines may be isolated to remove cell-produced cytokines that have little or no known treatment value in the selected indication or which are known or likely to have a negative impact on therapeutic efficacy. These determinations are made on the basis of existing clinical trial data, studies on cells in vitro, and information from the known literature on the actions of various cytokines. This information is partially summarized in Table 2.

Table 2. Cytokines: Biological Activities and Therapeutic Uses

Cytokine	Cellular sources	Biological Activities	Analysis & Applications [Potential/Approv. Clin. Uses]
INTERFERONS			
IFN-α- (>12 subtypes)	Mo/Mϕ, lymphoblast.	Antiviral & Anticancer	HBV, HCV, anti-Ca, HPV
IFN-β	fibroblast, Mϕ, epithelia	Immunomodulatory	Multiple sclerosis
IFN-γ	T-cell, NK cell	Immunomodulatory	CGD (chronic granulomat. dis)
TUMOR NECROSIS FACTORS			
TNF-α	Mo/Mϕ, fibroblast, T-cell	Cytotoxic, Apoptotic	Anticancer, too toxic by itself
sTNF-R	Mo/ Mϕ	Neutralizes TNF Anti-inflammatory	Rheumatoid Arthritis @ ? Sepsis, borderline efficacy
TNF-β (lymphotoxin)	T- & B-cell	? Neutralizes TNF	Insufficient data, skeptical
INTERLEUKINS			
IL-1α & IL-1β	Mo/Mϕ, endothelial	Immuno-stimulation	? Thombopoietic, ?anti-leukemia
	fibroblast, keratinocyte		Insufficient data
IL-1-ra	Mo/Mϕ	Blocks IL-1 binding	? Sepsis & Rheumatoid arthritis Skeptical
IL-2	T-cell	T-cell growth & function	Anticancer Rx., HIV @
IL-3	T- & mast cell	Multiple hematopoietic	T- & stem cell expansion ex vivo
		effects on BM stem cells >pleiotropic than G-CSF	Post-BM Transplant in vivo @ Bone marrow failure (chemoRx)
			Blood dyscrasia, Pancytopenia
IL-4	T- & mast cell	TH2 (humoral) stimulation ? Antigrowth	Stem cell expansion ex vivo Solid tumor
IL-5	T-cell, mast cell	Suppress allergis response ? supresses eosinophils	? Anti-asthma Rx, skeptical
IL-6	Mo/Mϕ, fibroblast, T-cell	Dual role in inflammation ? Antigrowth	? anti-inflammation ? anti-tumor, thrompoietic
IL-7	Bone marrow cell Keratinocytes	GF for preB cells Increases CTL, NK cells Cytokine synthesis in skin	Anti-melanoma @
IL-8	Mo, fibroblast, chondrocyte endothelia, keratinocyte pulmonary epithelia	CXC chemokine family Chemotactic Angiogenic	Antibacterial, antiviral Cancer patients @ Vessel growth, skeptical

IL-9	T-cell	Increases IgE synthesis	? Allergy treatment
IL-10	T-cell	Anti-inflammatory suppresses Langerhan cells	Trials: Inflam. Bowel Diseases Ulcerative colitis, Crohn's
IL-11	Bone marrow cell	Anti-inflammatory	Trials: anti-IBD Thrombopoietic @
IL-12 p35 & p40	B-cell, lymphoblastoid	NK stimulating factor	Solid tumor Rx.; @ Cancer patients,
immunotherapy			
IL-13	TH-2 cell	Ig E synthesis, allergenic ICAM, eosinophil growth	? Uses
IL-14 to IL-18	New cytokines, clinical applications to be defined		
COLONY STIMULATING FACTORS			
IL-3	T- & mast cell	Multiple hematopoietic	T- & stem cell expansion ex vivo
		effects on BM stem cells >pleiotropic than G-CSF	Post-BM Transplant in vivo @ Bone marrow failure
(chemoRx)			Blood dyscrasia, Pancytopenia
IL0-7			
G-CSF	Mo/Mϕ, fibrobl.,endothelia	Granulocyte maturation & oxidative bursts	Post-chemoRx pancytopenia Post-bone marrow transplant
GM-CSF	T-cell, fibrobl., endothelia	Granulocyte & Mϕ functions	Broader spectrum than G-CSF more side effects, same uses
M-CSF	Mo, fibroblast,endothelia	Monocyte growth	Similar to G-CSF
GROWTH FACTORS			
Epidermal growth factor	Mϕ	Cellular differentiation	Not applicable
TGF-α	Mϕ		Too toxic
TGF-β	platelet, Mo/Mϕ, T-, fibroblast, endothelia	Protects myocardium Antiinflammatory	? Heart attack, skeptical Too toxic
INTERCRINES			
Macrophage inflammatory proteins 1α , 1β (MIP)	Mo, fibroblast, T cell	Propagates inflammation	? Uses
Chemokines: RANTES	T-cell	Chemotactic	Immunocompromised host ? Uses
ANGIOGENIC FACTORS			
Fibroblast growth factors (FGF, acidic & basic)	platelet, Mϕ, endothelia	Angiogenesis Endothelial proliferation	Ischemic heart disease @ Congestive heart failure

Vascular endothelial Growth Factor (VEGF)	immune cells	Endothelial proliferation	Congestive heart failure
Platelet-derived Growth factors 1 & 2	platelet, Mo/Mϕ endothelia	Growth factor, angiogensis Indirect effects	Ischemic heart disease ? efficacy
ANTI-ANGIOGENIC FACTORS			
Angiostatin Endostatin	Immune cells	Anti-endothelial growth	Probably most potent anticancer cytokines with acceptable side effects @

Abbreviations: Mo, monocyte; M macrophages; Ca, cancer? questionable uses IL-1ra, IL-1 receptor antagonist; Rx, therapy; @ for further discussion Ca, cancer; CTL, cytotoxic T cells NK, natural killer cells; Rx, therapy;

One preferred anti-cancer or anti-tumor composition includes two and preferably three or more cytokines selected from among IL-1-alpha, IL-1-beta, IL-2, IL-4, IL-6, IL-12, IFN-alpha, IFN-beta, IFN-gamma, oncostatin, TNF-alpha, TNF-beta, GM-CSF, G-CSF, and M-CSF, more preferably selected from among IL-2, IL-12, IFN-alpha, IFN-beta, TNF-alpha, and GM-CSF. The composition is preferably treated to remove cytokine(s) selected from among IL-3, IL-5, IL-7, IL-8, IL-9, IL-10, IL-11, IL-1 and TGF-beta.

For use in treating viral infection, the composition preferably includes two or more cytokines selected from among IFN-alpha, IFN-beta, IFN-gamma, IL-3, IL-7, IL-8, IL-12, and GM-CSF, more preferably selected from among IFN-alpha, IFN-beta, IL-8, IL-12, and GM-CSF. The composition is preferably treated to remove cytokine(s) selected from among IL-1, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-11, IL-13, TNF-alpha, TNF-beta, TGF-beta, and oncostatin.

For use in treating an inflammatory condition, the composition preferably includes two or more cytokines selected from among IL-4, IL-5, IL-6, IL-10, IL-11, IL-13, and IFN-gamma, and preferably selected from among IL-4, IL-5, IL-6, and IL-10. The composition is preferably treated to remove from the composition, cytokine(s) selected from among IL-1, IL-2, IL-3, IL-7, IL-8, IL-9, IL-12, TNF-alpha, TNF-beta, TGF-beta, and oncostatin.

The compositions are used in cytokine therapy in treating cancer, viral infection, or inflammation according to standard therapy. In particular, the total cytokine dose administered is adjusted so that any one cytokine component is

administered at a lower dose, e.g., 10%-50% of the normal dose of that cytokine when administered alone.

Treatment Methods. The composition is administered by various systemic routes, e.g., intravenous, intramuscular, subcutaneous, transdermal, or by inhalation. Treatment is continued until a desired end point is reached, for example, reduction in tumor load, clearing of viral infection, or lessening of inflammation symptoms. Administration may be daily or 2-3 times/week. If no treatment benefit is observed after 1-2 weeks, the dose may be increased until some measure of treatment efficacy is observed and/or severe side effects interfere with patient comfort and compliance.

Specific examples of the steps described above are set forth in the following examples. However, it will be apparent to one of ordinary skill in the art that many modifications are possible and that the examples are provided for purposes of illustration only and are not limiting of the invention unless so specified.

Example 1

Preparation of Plasmids pEF-FLAG-Bcl-X_L and pcDNA-FLAG-PKR

A. Preparation of pEF-FLAG-Bcl-X_L

The pEF-FLAG-Bcl-X_L vector (Huang, et al., 1997) in Figure 1A contains a full length cDNA encoding the anti-apoptotic Bcl- X_L protein operably linked to the strong elongation factor 1alpha (EF-1alpha) promoter. An additional salient feature of the vector is the N-terminal FLAG epitope (Hopp et al., 1988) that was added to the Bcl-X_L protein to facilitate selection of cell lines that express high levels of Bcl-X_L.

The vector also includes i) a polyadenylation signal and transcription termination sequence to enhance mRNA stability; ii) a SV40 origin for episomal replication and simple vector rescue; iii) an ampicillin resistance gene and a ColE1 origin for selection and maintenance in E. coli; and iv) a puromycin resistance marker (Puro) to allow for selection and identification of the plasmid-containing eukaryotic cells after transfection of a Bcl-X_L and PKR.

B. Preparation of pcDNA-FLAG-PKR

The pcDNA-FLAG-PKR vector in Figure 1B contains cDNA encoding the full-length human PKR molecule (551 amino acids; Meurs, et al., 1990; GenBank Accession No. NM002759) modified by the polymerase chain reaction to include the N terminal FLAG tag (Hopp et al.,1988) encoding the sequence MDYKDDDDK, and inserted into the eukaryotic expression vector pcDNA3 (Invitrogen), such that the FLAG-PKR coding sequence was expressed under the control of the CMV promoter.

The vector, termed pcDNA-FLAG-PKR, contains various features suitable for PKR transcription, including: i) a promoter sequence from the immediate early gene of the human CMV for high level mRNA expression; ii) a polyadenylation signal and transcription termination sequence from the bovine growth hormone (BGH) gene to enhance mRNA stability; iii) a SV40 origin for episomal replication and simple vector rescue; iv) an ampicillin resistance gene and a ColE1 origin for selection and maintenance in E. coli; and v) a G418 resistance marker (Neo) to allow for selection and identification of the plasmid-containing eukaryotic cells after transfection.

Example 2

Preparation of PKR Over-producing Namalwa Cell Lines 6A and A9

1. Preparation of Cell Line 6A

The human B lymphoblastoid cell line Namalwa (WT) was transfected sequentially with the plasmids, pEF-FLAG-Bcl-X_L and pcDNA-FLAG-PKR. The transfected cell line is termed 6A.

Stable transfectants were obtained by electroporation of $4x10^6$ exponentially growing Namalwa cells with 15ug of the pEF-FLAG-Bcl-X_L plasmid in DMEM/F12 (+10% FBS) using a Gene Pulser apparatus (BioRad) set at 800 uF, 300V. Bulk populations of stable transformants were obtained by selection with 2 ug/ml puromycin (Gibco-BRL) for 3-4 weeks and screened for Bcl-X_L expression by flow cytometry as follows. The bulk transfectants were washed, permeabilized with acetone and subsequently stained with 2 ug/ml mouse anti-FLAG M2 monoclonal antibody (IBI) and then with phycoerythrin conjugated goat anti-mouse IgG (1ug/ml; Becton-Dickinson). Cells were analyzed in the

FACScan, live and dead cells being discriminated on the basis of their forward and side light-scattering properties and Bcl-X_L expressing cells by their level of fluorescence intensity. High level Bcl-X_L expressing transformants (Namalwa-Bcl-X_L) were then transfected with pcDNA-FLAG-PKR.

Stable high level Bcl-X_L expressing transfectants were obtained by electroporation of $4x10^6$ exponentially growing Namalwa- Bcl-X_L cells with 15 ug of the pcDNA-FLAG-PKR plasmid in DMEM/F12 (+10% FBS) using a Gene Pulser apparatus (BioRad) set at 800uF, 300V. Bulk populations of stable transformants were obtained by selection with 2 mg/ml geneticin (G418, Gibco-BRL) for 3-4 weeks. Clonal lines were subsequently obtained by limiting dilution cloning and analyzed for Bcl-X_L and PKR expression by Western blot analysis (Huang et al.,1997). The proteins were identified using 2 ug/ml anti-FLAG M2 antibody followed by goat anti-mouse IgG-peroxidase conjugate and ECL detection (Amersham).

B. Preparation of Cell Line A9

Stable high level PKR expressing transfectants were obtained by electroporation of $4x10^6$ exponentially growing Namalwa cells with 15 ug of the pTRE-PKR plasmid in DMEM/F12 (+10% FBS) using a Gene Pulser apparatus (BioRad) set at 800uF, 300V. Bulk populations of stable transformants were obtained by selection with 2 mg/ml geneticin (G418, Gibco-BRL) for 3-4 weeks. Clonal lines were subsequently obtained by limiting dilution cloning and analyzed for PKR expression by Western blot analysis (Huang et al.,1997).

Example 3

Culturing Cytokine-Producing Cells

Wildtype Nowalwa cells (WT) and the A9 and 6A cells from Example 2 were examined for cell viability in culture under conditions of PKR overproduction and cytokine induction. Specifically, PKR and Bcl-X_L double-transfected Namalwa cells (the 6A cell line), PKR-transfected Namalwa cells (the A9 cell line) and parental Namalwa cells (WT) were cultured at $2.5x10^5$ cells/ml in DMEM/F12 medium supplemented with 10% FBS. The cells were treated with 20 mM PMA (primer) for 20 hr followed by treatment with either 200 ug/ml poly r(I):poly r(C) and 10 ug/ml DEAE Dextran (poly IC induction) for 72 hr. or 200 HAU/$1x10^6$ cells

of Sendai virus for 48 hr. Following treatment, cell viability was assessed by flow cytometry on a FACScan.

Although the invention has been described with reference to particular embodiments and methods, it will be appreciated that various changes and modification may be made without departing from the invention as claimed.